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14. ABSTRACT

This study focuses on the identifying differences in the vasculature in the ends of long bones where breast cancer cells tend to lodge, as compared to the vasculature of the central marrow cavity. We have found differences in RNA expression by microarray analysis. The bone-derived vasculature expresses five RNA messages in greater abundance (2-fold or more) than the marrow-derived vasculation. Most of the more abundant RNA is related to angiogenesis. We have also found that the bone-derived cells express more surface protein (e-selectin) that can tether breast cancer cells. The expression of e-selectin is enhanced by secretions from early stage osteoblasts. This finding may help explain why breast cancer cells preferentially lodge in regions of bone where osteoblasts are abundantly present.

15. SUBJECT TERMS
Breast cancer cells, Adhesion Proteins, Trabecular bone, Osteoblasts

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Introduction

The goal of this research is to identify characteristics of the cellular environment in the ends of long bones that may foster the entrapment of metastasized breast cancer cells. More specifically, we are focusing on the vasculature for two reasons. One, studies published by others indicate that the endothelial cells in iliac crest biopsies express more surface adhesive proteins than other vascular endothelial cells (1). Two, the vasculature in regions where breast cancer cells lodge are the first tissue barrier the cancer cells encounter; this vascular network is unique with respect to blood flow and proximity to bone surfaces. We have developed a method to isolate endothelial cells from the ends of long bones and are comparing the bone vascular endothelial cells (BVECs) with marrow-derived vascular endothelial cells (MVECs) using immunodetection of surface adhesion molecules and microarray analysis.

Body

Task 1. Compare endothelial cells of the microvasculature from the ends of long bones with their counterparts from the central marrow cavity. The first task was to isolate vascular endothelial cells from the ends of long bone, where the vascular cells are close to and aligned with trabecular bone surfaces; these cells are designated as BVECs. At the same time, vascular cells were isolated from the central marrow cavity; these cells are called MVECs. After testing several cell isolation methods, we chose a magnetic bead method.

The endothelial cell isolation method we adopted after introducing a number of modifications in preliminary trials. Eleven female Swiss Webster mice, 7-9 weeks of age were euthanized by CO_2 inhalation. Tibias and femurs were removed and stripped of extraneous muscle and connective tissue. Bones were split longitudinally, exposing the central marrow cavity and metaphyseal region. Marrow was removed from the central third of the bone shaft and placed in DMEM (Sigma, St. Louis, MO) culture medium; remaining marrow in the shaft and metaphysis was flushed out and discarded. Metaphyseal bone was then scraped with a No. 1 curette into DMEM to obtain trabecular bone fragments with adherent vascular endothelial cells.

The metaphyseal (BVEC) and marrow (MVEC) isolates were treated with 0.1% collagenase for 30 minutes at 37°C. Cell preparations were centrifuged at 1200 rpm and resuspended in Medium 199 (MediaTech, Herndon, VA) supplemented with 20% fetal bovine serum (Sigma), 1% penicillin/streptomycin (MediaTech) and 1X endothelial cell growth factor (Sigma). Cells were plated in 60mm tissue culture dishes coated with 2% gelatin and incubated at 37°C with 5% CO_2 humidified atmosphere. Cells were cultured for 7 days; media was changed every other day.

Endothelial cells were separated from other contaminating cell types using the Miltenyi VarioMACS magnetic cell sorting system (Auburn, CA). After one week, BVEC and MVEC cultures were harvested with the cell dispersion solution Accutase (Innovative Cell Technologies, San Diego, CA) and resuspended at a density not exceeding $5X10^6$ cells/500µl in a labeling buffer consisting of PBS with 2mM EDTA. The isolectin B4 from *Griffonia (Bandeirea) simplicifolia* seeds binds preferentially to mouse endothelial cells (2) and so was used to label the endothelial cells for magnetic sorting. The biotinylated isolectin B4 (Vector Laboratories, Burlingame, CA) was diluted 1:50 in the each cell suspension and incubated at 4°C

for 30 minutes. Cells were washed twice with labeling buffer then resuspended in 90µl of labeling buffer. Magnetic microbeads coated with streptavidin (Miltenyi) were added to the labeled cell suspension and incubated at 4° for 15 minutes. Cells were washed twice with labeling buffer and resuspended in 500µl of separation buffer consisting of PBS with 2mM EDTA and 0.5% calf serum. A MACS separation column was prewashed with separation buffer and placed in the VarioMACS magnetic stand. A cell suspension was then loaded into the reservoir of each column and allowed to flow through the column. Non-endothelial cells that were not labeled with the biotinylated isolectin lacked streptavidin coated magnetic microbeads attached to their surfaces and thus would not be retained in the column. All columns were rinsed twice with separation buffer then removed from the magnetic stand. Magnetically labeled endothelial cells retained in the column were then flushed out in 1 ml of separation buffer. Cells suspensions were centrifuged at 1200 rpm and resuspended in Medium 199 supplemented with 20% FBS, 1% penicillin/streptomycin and 1X endothelial cell growth factor (ECGF). Cells were plated at a density of approximately 50,000 cells/cm^2 in gelatin coated 35 mm plates and incubated at 37°C for 7 days with media changes every other day. Cultures of putative endothelial cells isolated by this method were checked for purity by assessing their ability to take up fluorescent labeled acetylated LDL at an accelerated rate (3).

After one week in culture BVECs and MVECs were harvested. Total RNA was extracted from both cell types using the Qiagen RNeasy kit (Valencia, CA). Microarray labeling, hybridization and analysis was conducted on the RNA by the Penn State University Microarray Facility under the direction of Dr. Craig Praul. Briefly, RNA obtained from three separate isolations of BVECs and MVECs was labeled using the Affymetrix GeneChip Expression 3' Amplification One-Cycle Target Labeling kit (Santa Clara, CA). After biotin labeling of the antisense strand, generated cRNAs were fragmented and hybridized to the Affymetrix GeneChip Mouse Genome 430A 2.0 array. Quality of the starting total RNA, the intact cRNA and the fragmented cRNA were assessed with the Agilent Bioanalyzer (Palo Alto, CA) and RNA 6000 Nano Lab chip. All RNAs at all stages were deemed of superior quality for analysis. Arrays were read by the Affymetrix GeneChip scanner and interpreted by GeneChip Operating software version 1.3.

Microarray data analysis was performed by Qing Zhang at the Penn State University Bioinformatics Consulting Center. Fold increase or decrease values were determined by comparing expression levels of BVECs to the expression levels of MVECs averaged from three isolations, as shown in the table below.

Table I. Differences in mRNA expression in BVECs and MVECs

Affymetrix #	Gene name	BVEC fold change over MVEC
1418752_at	Aldehyde dehydrogenase	+3.9
1420450_at	Matrix metalloproteinase 10	+2.7
1417256_at	Matrix metalloproteinase 13	+2.5
1427760_s_at	Proliferin	+2.2
1415935_at	SPARC-related calcium binding (SMOC-2)	+2.1
1449193_at	CD5-like antigen	-3.1
1450407_a_at	Acidic nuclear phosphoprotein 32	-2.2
1419519_at	Insulin-like growth factor 1	-2.1
1418847_at	Arginase type II	-2.0

These data support our hypothesis that bone-derived vascular endothelial cells (BVECs) have unique properties when compared to control cells (MVECs). Interestingly, four of the proteins expressed in greater abundance by the BVECs are proteins that are involved in angiogenesis.

Task 2. Determine the difference in attraction of the breast cancer cells to the two types of vascular cells. Based on studies done by Lehr and Pienta (1), we postulated that the surface adhesion molecules p-selectin, e-selectin, ICAM and VCAM are expressed constitutively on the endothelial cells associated with trabecular bone and that these molecules could serve to tether metastasizing breast cancer cells to the endothelium and aid in extravasation. Initially, we expected to see that BVECs expressed these adhesion molecules at a higher frequency than MVECs. In our first experiments we isolated BVECs and MVECs as in task 1 and stained them for the presence of surface p-selectin, e-selectin, ICAM and VCAM. We found no significant differences in adhesion molecule expression between the two cell types (Figure 1).

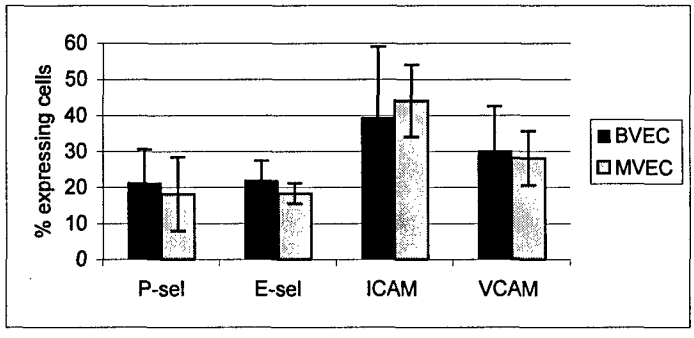

Figure 1. Basal levels of surface expression of tethering (p-selectin, e-selectin) and adhesive (ICAM, VCAM) proteins by BVECs and MVECs.

Because no differences were found, we then hypothesized that a neighboring cell type, specifically osteoblasts, may be responsible for the upregulation of adhesion molecules on the surface of the adjacent endothelial cells. Osteoblasts lining trabecular bone in the metaphysis are numerous and are located in close proximity to the BVECs; MVECs in the central marrow cavity, on the other hand, are almost never found close to osteoblasts. Osteoblasts are known to secrete such proteins as TGFβ and VEGF, which could influence the presentation of adhesion molecules on the surface of the endothelial cells (4,5).

Three separate isolations of MVECs and BVECs were cultured 7 days in Medium 199 supplemented with bovine serum and endothelial cell growth factor. The vascular cells were then exposed to conditioned medium derived from mouse osteoblast cell line (MC3T3-E1) for 24 hours. Since secretions of osteoblasts vary according to their stage of development, conditioned media from both immature (5-7 day culture) and mature (29-31 day culture) osteoblast cultures were used. To demonstrate adhesion molecules on the cell surfaces, BVECs and MVECs were exposed to antibodies against p-selectin, e-selectin, ICAM-1 and VCAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA). A fluorescent secondary antibody (Molecular Probes, Eugene,

OR) was applied, then cells were thoroughly rinsed and fixed. The cells were visualized and counted using confocal microscopy. As shown in Figure 2, MVECs treated with medium from immature osteoblasts or medium from mature osteoblasts were not significantly different from each other or from the control treatment.

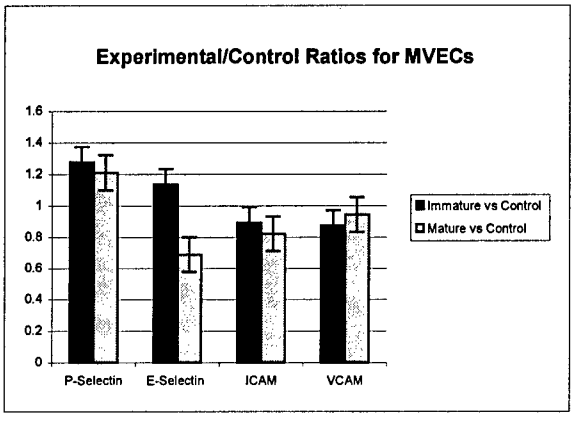

Figure 2. MVECs treated with osteoblast secretions. No significant responses were found.

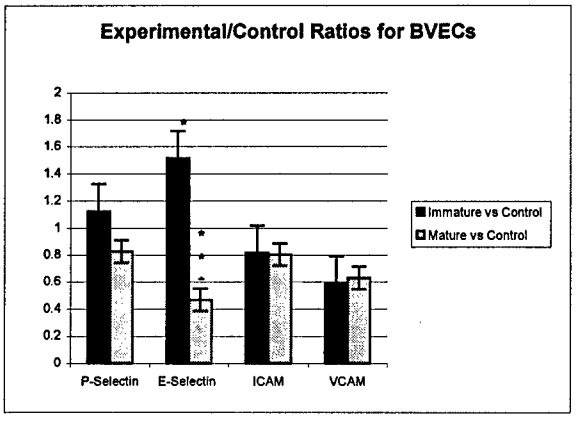

Figure 3. BVECs treated with osteoblast secretions. Surface expression of e-selectin was significantly altered.

BVECs responded in a differential manner (Figure 3). E-selectin was present on 33% more BVECs when treated with *immature* osteoblasts CM when compared to control media, E-selectin was lower by 53% when treated with *mature* osteoblast CM. The data suggest that *in vivo* endothelial cells juxtaposed to immature osteoblasts are upregulated for surface expression of e-selectin and may participate in tethering cancer cells in the blood stream at a higher frequency than other cells. This differential response of BVECs to osteoblast secretions supports our hypothesis that there are inherent differences between endothelial cells of the central marrow cavity and those of the metaphysis, a site of preferential metastasis for breast cancer cells.

Key Research Accomplishments

1. Improved the endothelial cell isolation method by employing magnetic sorting technology.
2. Obtained high quality RNA from BVECs and MVECs.
3. Compared BVEC and MVEC RNA by microarray analysis and found 9 proteins differing by 2-fold or more.
4. Found that secretions from immature osteoblasts stimulate BVECs to express surface e-selectin and that MVECs were unresponsive to both types of osteoblast secretions. This finding may help explain why breast cancer cells most frequently metastasize in bone.

Reportable Outcomes

1. Makuch, L. "Specificity of Breast Cancer Cells for Bone: The effects of osteoblast secretions on vascular endothelial cells," Honors Thesis, The Pennsylvania State University, 2005. [This work is currently being prepared for publication.]
2. Gay, C.V., Makuch, L.A., Geffel, D.L. and Sosnoski, D.M., "Properties of well-vascularized regions of bones that become colonized by breast cancer cells," Era of Hope Conference, 2005 (abstract).
3. Gay, C.V., Makuch, L.A., Geffel, D.L. and Sosnoski, D.M., "Enhanced expression of E-selectin on bone-derived vascular endothelial cells: Potential role in breast cancer metastasis," Am. Soc. for Bone & Mineral Research Annual Meeting, 2005.

Conclusions

We have identified differences in RNA expression between bone-derived vascular endothelial cells (BVECs) and marrow-derived vascular endothelial cells. The BVECs express more RNA related to angiogenesis than do MVECs.

Secondly, we have shown that osteoblast secretions stimulate the expression of more tethering proteins (e-selectin) on BVECs and that MVECs were unresponsive. Breast cancer cells are known to possess surface ligands for e-selectin. This finding may help explain why breast cancer cells preferentially lodge in the ends of long bones and other bones rich in trabecular bone.

References

1. Lehr, J.E. and Pienta, K.J. Preferential adhesion of prostate cancer clels to a human bone marrow endothelial cell line. J. Natl. Cancer Inst. 90(2):118-123, 1998.

2. Marelli-Berg, F.M., Peek, E., Lidington, E.A., Stauss, H.J., and Lechler, R.I. Isolation of endothelial cells from murine tissue. J. Immuno. Meth. 244:205-215, 2000.

3. Voyta, J.C., Via, D.P., Butterfield, C.E., and Zetter, B.R. Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. J. Cell. Biol. 99:2034-2040, 1984.

4. Kang, Y.H., Brummel, S.E. and Lee, C.H. Differential effects of transforming growth factor-beta 1 on lipopolysaccharide induction of endothelial adhesion molecules. Shock 6(2):118-125, 1996.

5. Kim, I., Moon, S-O, Kim, S.H., Kim, H.J., Koh, Y.S., and Koh, G.Y. and Koh, G.Y. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and e-selectin through nuclear factor-kappa b activation in endothelial cells. J. Biol. Chem. 276(10):7614-7614, 2001.